



Minireview

How is ferredoxin-NADP reductase involved in the NADP photoreduction of chloroplasts?*

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Abstract

NADP photoreduction of chloroplasts was discovered in 1951, and subsequent research was conducted to elucidate the enzymatic mechanisms involved in this reaction. In 1963, ferredoxin-NADP reductase (FNR; EC 1.18.1.2, ferredoxin-NADP oxidoreductase) was isolated and purified to a crystalline form. Because the reaction mechanism of ferredoxin-NADP reducing system was clarified in the isolated enzyme system, it was generally thought that the role of FNR in the NADP photoreduction of chloroplasts had been fully elucidated. However, the results of a reconstitution study using the crystallized FNR and the depleted grana, from which ‘built-in’ FNR had been eliminated, showed that the NADP photoreducing activity of reconstituted FNR was much lower than the original physiological activity, and as a result, more studies had to be continued. In 1985, a protein factor called ‘connectein’ was discovered, and it was shown that this new protein binds with two FNR molecules to form an FNR–connectein complex. Then in 1991, the FNR–connectein complex was formed using purified connectein and FNR, and after eliminating ‘built-in’ FNR, the reconstituted complex was bound to the depleted grana having reduced NADP photoreducing activity. The results showed that NADP photoreducing activity of the reconstituted system was comparable to the original physiological activity. This proved that the FNR–connectein complex, which binds to a specific site on the surface of thylakoid membrane, is functionally responsible for NADP photoreduction in chloroplasts.

Abbreviations: FNR – ferredoxin-NADP reductase; NADP – nicotinamide adenine dinucleotide phosphate; PPNR – photosynthetic pyridine nucleotide reductase (a former name for ferredoxin); TPN – triphosphopyridine nucleotide (a former name for NADP)

Background

In 1951, three laboratories independently reported that, when illuminated, ‘free’ chloroplasts were capable of generating oxygen and reducing NADP (Arnon 1951; Tolmach 1951; Vishniac and Ochoa 1951). This was the beginning of enzymatic research

on NADP photoreduction in chloroplasts. Later, three protein factors were isolated: methemoglobin reducing factor, which is needed for utilizing methemoglobin as a Hill oxidant (Davenport et al. 1952); TPN reducing factor, which is a functional catalyst for photosynthetic phosphorylation (Arnon et al. 1957); and photosynthetic pyridine nucleotide reductase (PPNR), which is catalytically active for pyridine nucleotide reduction in illuminated chloroplasts (San Pietro and Lang 1958).

*The present article is dedicated to the memory of Professor Dr. Daniel I. Arnon (1905–1993).

The results of subsequent comparative studies clarified that these three soluble protein factors were the same chemical compound, and they were collectively referred to as PPNR. With regard to quantification of NADP photoreduction, in 1951, NADP photoreduction was quantified indirectly by measuring products formed by coupling the dehydrogenase system, which consumes reduced pyridine nucleotide. San Pietro and Lang (1956) later developed a method that could directly quantify reduced pyridine nucleotide accumulated in a reaction mixture by a spectrophotometric analysis. They observed that illuminated grana were better able to reduce NAD than NADP, and then isolated the following enzymes from spinach: PPNR, the enzyme required for the NADP photoreduction of illuminated grana; and transhydrogenase, the enzyme that transfers hydrogen from NADPH, formed by PPNR, to NAD (Keister et al. 1960). This was the basis of 'San Pietro's transhydrogenase theory,' which states that illuminated grana reduce NADP in the presence of PPNR, and transhydrogenase then reduces NAD using reduced NADP.

Discovery of ferredoxin-NADP reductase (FNR) in Berkeley, California

In 1962, Tagawa and Arnon compared PPNR and a new electron carrier called 'ferredoxin,' which was discovered in the hydrogenase system of *Clostridium pasteurianum* (Mortenson et al. 1962), and revealed that while one was involved in bacterial hydrogen generation and the other in higher plant NADP photoreduction, they were functionally similar and were interchangeable non-heme-iron proteins. Until this time, PPNR was viewed as a reductase, but because it was shown to be an electron carrier called ferredoxin, research was conducted to identify the 'true reductase,' responsible for electron transfer from photosynthetically reduced ferredoxin to NADP. In 1963, Shin et al. subjected a spinach leaf extract to acetone fractionation, ammonium sulfate fractionation, and ion-exchange column chromatography using DEAE-cellulose, and then purified a golden-colored crystalline flavoprotein based on crystallization in the ammonium sulfate solution (Figure 1). This flavoprotein reduced cytochrome c from NADPH via ferredoxin, and it was thus believed that the flavoprotein would catalyze the opposite reaction from ferredoxin to NADP in illuminated chloroplasts. Figure 2 shows the results of a reconstitution

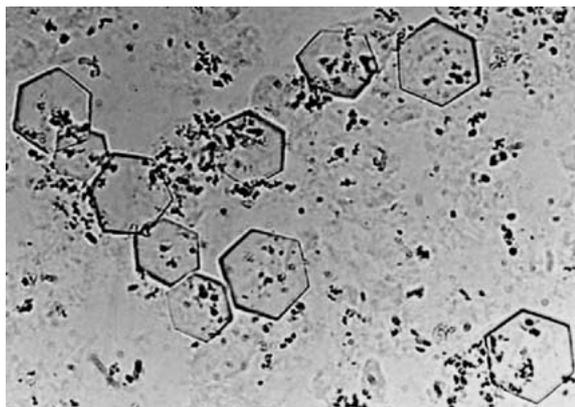


Figure 1. Hexagonal crystals of ferredoxin-NADP reductase (FNR) from spinach (unpublished photograph by M. Shin, taken in 1968).

study that was conducted to investigate this point. Illuminated grana are able to reduce NADP upon addition of ferredoxin because the 'true reductase' is bound as a 'built-in' enzyme in the membrane. As a result, a special extraction procedure was employed to remove this 'built-in' enzyme, and the results showed that while the activity of the depleted grana was low when ferredoxin was added, it increased with addition of the crystallized enzyme in a dose-dependent manner. This clarified that both ferredoxin and flavoprotein were involved in NADP photoreduction. Davenport (1963) independently came to the same conclusion when he conducted a reconstitution test using pea chloroplasts as an extension of his research on methemoglobin reducing factor. The absorption spectrum of crystalline FNR is that of a typical flavoprotein. Changes in absorption show that this enzyme is reduced by reduced ferredoxin, and oxidized by NADP. Using pyridine nucleotides, the K_m value for NADP was 7.72×10^{-6} M and for NAD 3.77×10^{-3} M, and thus, in physiological concentrations of pyridine nucleotides in chloroplasts, only NADP can be reduced (Shin and Arnon 1965). The possibility of regulating electron flow in the photosynthetic electron transfer system (Nelson and Neumann 1969) by forming a stable complex with a 1:1 ratio for ferredoxin and FNR (Shin and San Pietro 1968; Foust et al. 1969; Nelson and Neumann 1969) was investigated, but complex formation itself was shown to be dependent entirely on the conformation of the two proteins, and independent of electron transfer between them (Shin and Sakihama 1984). FNR reduces numerous physiological and non-physiological substances having NADPH as an electron donor, and has been studied and described in terms of the different reactions it

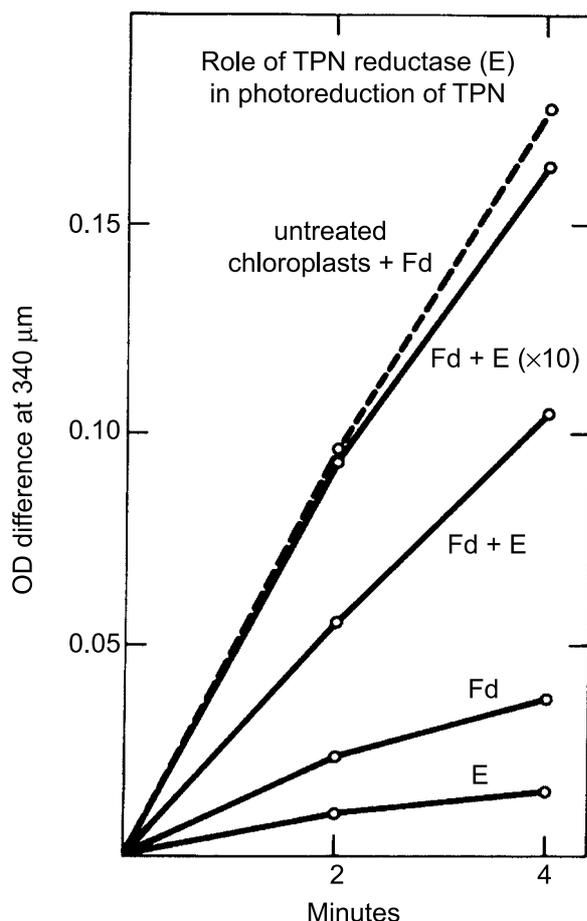


Figure 2. The role of FNR in NADP photoreduction of chloroplasts (Shin et al. 1963; original slide presented at the annual meeting of the Federation of American Societies of Biological Chemists in 1963). Spinach chloroplasts were prepared by the method of Whatley and Arnon (1963). From the untreated chloroplasts, 'built-in' FNR was released in 0.05 M Tris-HCl buffer, pH 7.6, by overnight incubation and the supernatant was then separated by centrifugation. Photoreduction of NADP labeled then as TPN was measured by the absorbance change at 340 nm under the illumination of red actinic light. The reaction mixture contained NADP, ascorbate-indophenol dye couple as an artificial electron donor and, in broken line, 'untreated chloroplasts' or, in solid line, 'treated grana.' Additions are shown on the lines; Fd, ferredoxin; E, crystallized FNR in the same amount of 'built-in FNR'.

will catalyze. For example, before the FNR function of the flavoprotein was identified, the indophenol reducing activity of TPNH diaphorase was studied by Avron and Jagendorf (1956), and the NAD reducing activity of transhydrogenase was investigated by Keister et al. (1960). In addition, 'Gelben ferment' and 'Roten ferment' isolated from *Chlorella* (Gewitz and Völker 1962) correspond to FNR and ferredoxin, respectively. Even after the FNR function of the flavoprotein was identified, the flavoprotein was designated

as ferredoxin-cytochrome *f* reductase (Zanetti and Forti 1966).

Discovery of connectein in Kobe, Japan

Ever since Avron and Jagendorf (1957) first documented the molecular weight of the flavoprotein, values from 32 to 117.5 kDa were reported up to about 1978 (Zanetti and Forti 1966; Keirns and Wang 1972; Schneeman and Krogmann 1975; Fredricks and Gehl 1976; Gozzer et al. 1977; Bookjans et al. 1978; Hasumi and Nakamura 1978). Several compounds were occasionally seen in purified flavoprotein, and the cause of this molecular heterogeneity was debated for some time (Ellefson and Krogmann 1979; Sheriff et al. 1980; Hasumi et al. 1983). In 1984, Karplus et al. elucidated the entire primary structure of the flavoprotein, and its molecular weight was found to be 35 kDa. Because the last 18 amino acid residues on the N-terminus are likely to be subjected to enzymatic degradation during purification and storage, the molecular weight of FNR can gradually decrease to 33 kDa without any functional decline, thus contributing to its molecular heterogeneity (Shin et al. 1990). Therefore, values of 32–50 kDa represent the molecular weight of FNR, which is originally 35 kDa. However, values of 85–117.5 kDa can only be explained by the presence of a larger form of FNR. When FNR was extracted from chloroplasts using ammonium sulfate at high-concentration (40% saturation) and then subjected to ammonium sulfate fractionation, gel-filtration chromatography on a Sephadex column gave two FNR peaks (Fredricks and Gehl 1976; Shin and Oshino 1980; Zanetti and Arosio 1980; Shin et al. 1981). While the slowly eluting peak contained the conventional FNR, the fast-eluting peak contained a larger form of FNR. This large-form FNR was shown to be unstable under low ionic strength conditions, and purification under high ionic strength conditions revealed that this FNR had a molecular weight of 75 kDa (Yamasaki et al. 1983). Interestingly, when this 75 kDa FNR was dissolved under low ionic strength conditions, an FNR with slightly higher molecular weights from 33 to 51 kDa, was obtained, but when the dissolved FNR was placed in high ionic strength conditions, the 75 kDa FNR was again formed. However, this ability to reform the 75 kDa FNR was lost when FNR was highly purified into the 33 kDa FNR. Following these observations, we predicted that the 75 kDa FNR was not a homodimer, but rather was a protein complex having two FNR molecules and an

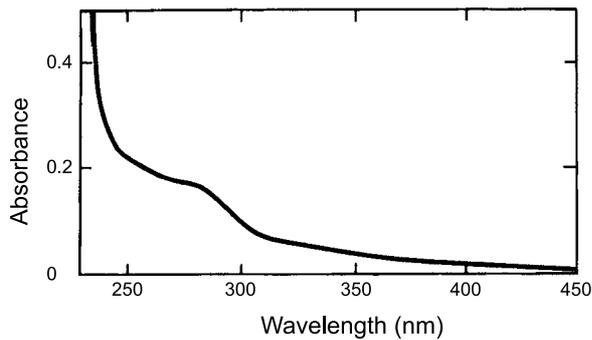


Figure 3. Absorption spectrum of connectein from spinach (after Shin et al. 1985).

unknown factor. Shin et al. (1985) later discovered that, when isopropyl alcohol was added to purified 75 kDa FNR, 33 kDa FNR formed a precipitate, and the unknown factor was dissolved in isopropyl alcohol. When this unknown factor was highly purified and added to the 33 kDa FNR under high ionic strength conditions, the 75 kDa FNR was reformed. This factor was named 'connectein' because it was responsible for connecting two FNR molecules. The highly purified unknown factor was a colorless protein without remarkable absorption peaks at 280 nm (Figure 3), and its molecular weight, as measured by gel filtration, was about 10 kDa. The connectein was shown to be highly heat stable and its function was not lost when heated at 80 °C for 2 h. Connectein contained numerous proline residues (17.34%), serine (12.27%) and glutamic acid (11.08%), but phenylalanine, histidine and arginine were not detected (Y. Nozaki, unpublished data from 1985). However, it cannot be stained by either Coomassie brilliant blue or silver stain, and thus polyacrylamide gel electrophoresis has not yet yielded any useful information (Shin 1990). As a result, the primary structure of connectein has yet to be elucidated.

Reconstitution of the NADP photoreducing system using FNR–connectein complex

In order to prove the physiological function of FNR, a reconstitution experiment conducted in 1963 is shown in Figure 2. The FNR used in this experiment probably existed in its soluble state in the reaction mixture. Even when the same amount of crystallized FNR was added to replace FNR that was removed, the original activity could not be restored; restoration of the original activity required 10 times the amount of

eliminated FNR. Because FNR is an enzyme bound to the thylakoid membrane, it is necessary to study this enzyme in its bound state in order to investigate its true physiological function. Nozaki et al. (1985) investigated the connection between FNR and the thylakoid membrane by trypsin-based extraction (Forti et al. 1983), and documented that the FNR–connectein complex accounted for the majority of extracted FNR. This suggested that FNR is bound to the surface of thylakoid membrane via connectein. Therefore, Nakatani and Shin (1991) used highly purified 33 kDa FNR and connectein in order to form FNR–connectein complexes and incubated these with the depleted grana in an isotonic medium containing 0.35 M NaCl. After incubation for 3 h at 4 °C, the grana fraction was collected and examined to see whether its enzymatic activities were restored. As clearly shown in Figure 4, the depleted grana recovered NADP photoreducing activity to 93%, while NADPH diaphorase activity recovered to 61%. Even when only the 33 kDa FNR was incubated with the depleted

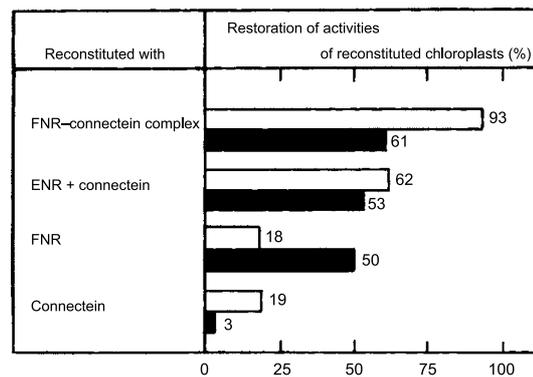


Figure 4. Restoration of enzymatic activities of the depleted grana by rebinding of FNR–connectein complex. The original data of Nakatani and Shin (1991) were adapted for this figure. The depleted grana were prepared from whole chloroplasts by incubation in 20 mM Tris-HCl buffer, pH 7.8 for 60 min at 0 °C, and by separating them from the supernatant by centrifugation. This quick extraction selectively released 'the loosely bound FNR' from chloroplasts and 'the tightly bound FNR' remained on thylakoids. After the extraction, enzymatic activities (NADP photoreducing and NADPH diaphorase activities) were diminished to about 2/3 of the original activities. Difference in enzymatic activities between whole chloroplasts and 'depleted grana' was taken as 100% standard for restoration. The FNR–connectein complex used was prepared from purified FNR and connectein by incubating their mixture for 3 h at 4 °C in an hypertonic medium containing 1 M NaCl. After incubation, the mixture was mixed with 'the depleted grana' in an isotonic medium containing 0.35 M NaCl and incubated for 3 h at 4 °C in order to make the reconstituted chloroplasts. The reconstituted chloroplasts were collected by low speed centrifugation at 6000 × g and examined their enzymatic activities.

grana, FNR also bound to the grana and NADPH diaphorase activity recovered to almost the same level (50%), but NADP photoreducing activity recovered to only 18%. When reconstituted with a mixture of FNR and connectein, almost the same amount of FNR bound to the depleted grana, judging from restoration of NADPH diaphorase activity of 53%, and NADP photoreducing activity was strongly enhanced to 62%. Therefore, connectein appeared to have guided FNR to its correct position on the thylakoid membrane, although FNR itself bound to thylakoid membrane in the presence or absence of connectein. In order to take back the FNR–connectein complex to the depleted grana safely, a reaction medium containing 0.35 M NaCl had to be used. The isotonic medium that Daniel Arnon recommended for chloroplast preparation also included 0.35 M NaCl (Whatley and Arnon 1963). In other words, the salt conditions that were established to prepare vital chloroplasts were also useful for stably binding FNR–connectein complexes to thylakoid membrane.

Concluding remarks

Based on the above-mentioned experimental findings, a schematic view of the ferredoxin-NADP reducing

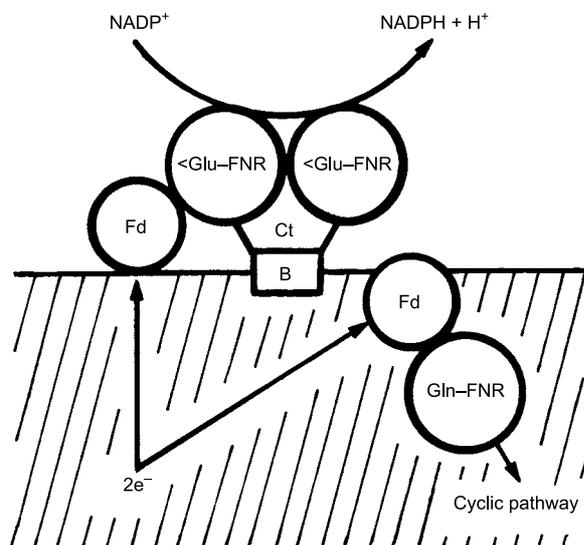


Figure 5. Arrangement of the FNR–connectein complex in NADP photoreducing system on thylakoid membrane. Glu-FNR and Gln-FNR, FNR with γ -pyroglutamic acid and with glutamine at the N-terminus, respectively (Sakihama et al. 1995); Ct – connectein; B – base protein (Nozaki et al. 1985); and Fd – ferredoxin.

system of the thylakoid membrane, the final step of the photosynthetic electron transport system, is shown in Figure 5. The FNR–connectein complex, consisting of two FNR and one connectein molecules, is bound to a trypsin-digestible base protein, which is believed to exist on the surface of thylakoid membrane (Nozaki et al. 1985). Ferredoxin is probably adjacent to FNR or is part of an FNR–ferredoxin complex to facilitate electron transfer from Photosystem I to NADP. The important point here is that there are two types of FNR: while one type of FNR participates in the ferredoxin-NADP reducing system by loosely binding on the surface of thylakoid membrane, the other type is strongly anchored in the membrane (Matthijs et al. 1986; Nisikawa et al. 1992). We have documented structural difference in the N-terminal residue between these two types of FNR with different binding modalities: the loosely bound FNR possessed γ -pyroglutamic acid at its N-terminal end and the tightly bound FNR glutamine (Sakihama et al. 1995). For some time, tightly bound FNR has been thought to be involved in the cyclic pathway (Shahak et al. 1981), but this issue is beyond the scope of the present Minireview.

In order to relate the story of this paper to Photosystem I (PS I), in general, I refer the readers to historical papers by Nelson and Ben-Shem (2002), Buchanan et al. (2002), and Ke (2002) for discussions on PS I, ferredoxin/thioredoxin system and P430 (an acceptor of PS I), respectively. A photograph of Daniel Arnon appears on p. 150 of Porra et al. (2002), of Mordhay Avron on p. 234 of Jagendorf (2002), and of Robert Hill on p. 51 of Walker (2002). Figure 6

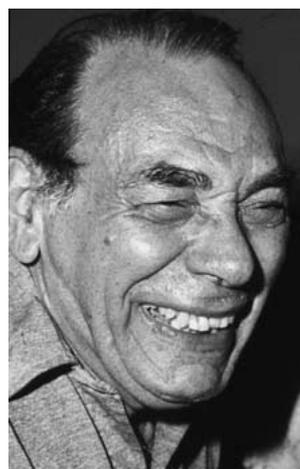


Figure 6. A photograph of Tony San Pietro. Date and place unknown. Photo was provided by Govindjee.



Figure 7. The author, Masateru Shin, taken in Kobe, Japan, in 1987.

is a photograph of Tony San Pietro and Figure 7 is a photograph of the author.

As mentioned in the footnote on p. 307, this paper is dedicated to the memory of Dr. Daniel I. Arnon. Melis and Buchanan (1995) provided several tributes to him in a 376-page special issue of *Photosynthesis Research*.

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